RESEARCH *New Phytol.* (2000), **146**, 211–217

Dynamics of Ni-based defence and organic defences in the Ni hyperaccumulator, *Streptanthus polygaloides* (Brassicaceae)

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Received 13 September 1999; accepted 13 January 2000

SUMMARY

Plants use chemical defences to reduce damage from herbivores and the effectiveness of these defences can be altered by biotic and abiotic factors, such as herbivory and soil resource availability. *Streptanthus polygaloides*, a nickel (Ni) hyperaccumulator, possesses both Ni-based defences and organic defences (glucosinolates), but the extent to which these defences interact and respond to environmental conditions is unknown. *S. polygaloides* plants were grown on high-Ni and low-Ni soil and concentrations of Ni and glucosinolates were compared with those of the congeneric non-hyperaccumulator, *S. insignus* spp. *insignus*, grown under the same conditions. Ni contents were highest (4000 μg g^{-1} dry tissue) in *S. polygaloides* plants grown on high-Ni soil. Glucosinolate content was significantly higher in *S. insignus* than in *S. polygaloides* suggesting that plants defended by Ni produce a lower concentration of organic defences. In a separate experiment, high-Ni *S. polygaloides* plants were exposed to simulated herbivory or live folivores to determine the inducibility of Ni-based and organic defences. Contents of Ni were not affected by either herbivory treatment, whereas glucosinolate concentrations were >30% higher in damaged plants. We concluded that the Ni-based defence of *S. polygaloides* is not induced by herbivory.

Key words: Ni hyperaccumulation, herbivory, elemental defence, *Streptanthus*, glucosinolates, serpentine, induced defence.

INTRODUCTION

Plants are defended from herbivory by a diverse arsenal containing both physical (e.g. spines, sclerified tissues) and chemical defences. Plant chemical defences can be characterized as either organic compounds synthesized from photoassimilates (e.g. tannins, glucosinolates) or as inorganic compounds sequestered from the soil (e.g. metals, silica). The latter, 'elemental' defences, include silicification (McNaughton & Tarrants, 1983), fluoroacetate (Twigg & King, 1991), calcification (Pennings & Paul, 1992; Hay *et al*., 1994), and hyperaccumulated metals (Boyd, 1998). Many elemental defences are bound to organic ligands, usually small organic acids (Twigg & King, 1991; Krämer *et al*., 1996; Sagner *et al*., 1998), but it is their inorganic components (e.g. fluorine, nickel, zinc) that confer antiherbivore capabilities.

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Metal-hyperaccumulating plants translocate remarkably high concentrations of metals from the root–soil interface and sequester those metals in aboveground tissues. Baker & Brooks (1989) defined metal hyperaccumulators as plants containing >1000 μg g^{-1} d. wt of cobalt (Co), copper (Cu), chromium (Cr), lead (Pb) or Ni or >10000 μg g^{-1} d. wt of manganese (Mn) or zinc (Zn) in their tissues. Although the selective value of metal hyperaccumulation has not been fully determined (Boyd & Martens, 1992, 1998), most experimental evidence to date suggests that hyperaccumulated metals have a defensive function against herbivory (Boyd, 1998). Elevated foliar metal concentrations can negatively affect herbivores by causing acute toxicity (Boyd & Martens, 1994; Martens & Boyd, 1994; Boyd & Moar, 1999), prolonging larval development (Boyd & Moar, 1999) and deterring feeding (Pollard & Baker, 1997).

The range of concentrations of accumulated metal(s) within the leaves of many hyper-

accumulators has been well documented (see Reeves *et al.*, 1996, 1999; Brooks, 1998; Boyd *et al.*, 1999). The dynamics of metal hyperaccumulation, however, have been largely neglected, especially within the context of plant defence. The contents of many organic plant defences fluctuate in response to plant age (Coley & Aide, 1991), resource availability (Coley *et al.*, 1985; Bryant *et al.*, 1987) and herbivore damage (Karban & Baldwin, 1997). Although some studies have addressed the effects of age (Kruckeberg & Reeves, 1995; Boyd *et al.*, 1999) and resource availability (Brown *et al.*, 1995a,b) on the metal contents of hyperaccumulators, none has examined the role that herbivores might play in determining concentrations of defensive metals.

Herbivores can greatly influence the contents of certain organic defences within plants (Karban & Baldwin, 1997). Variation in the degree to which plant defences respond to herbivory has led to the classification of plant defences as either constitutive or inducible (Howe & Westley, 1988). Large compounds (e.g. tannins) which are metabolically expensive to synthesize are often considered to be constitutive defences. Relatively small compounds that are synthesized or activated in direct response to herbivore damage (e.g. glucosinolates, cyanogenic glycosides) are categorized as inducible defences. With the exception of McNaughton & Tarrants (1983), no studies have attempted to place elemental plant defences into either category. McNaughton & Tarrants (1983) showed that silica contents of several African savanna grass species increased after exposure to simulated herbivory treatments; thus, they considered silicification to be an inducible defence. The response of foliar metal concentrations of hyperaccumulators to herbivore damage has not been determined.

Plants often use a combination of defences to reduce herbivory and the levels of these defences within plants respond to many environmental factors. Few studies, however, have examined multiple defences within a plant species, and therefore the interactive effects (i.e. synergisms or antagonisms) of most plant defences are unknown (Duffy & Paul, 1992; Pennings, 1996; Pennings *et al.*, 1998). Martens & Boyd (1994) speculated that plants that are well defended by foliar metal should invest less carbon in the construction of organic defences than plants that do not use a metal-based defence. Therefore, we expected to find lower concentrations of organic defences in a Ni hyperaccumulator than in a congeneric non-hyperaccumulator. Likewise, when Ni hyperaccumulators are grown on low-Ni soil, thereby limiting the availability of Ni-based defences, we expected to find higher concentrations of organic defences than in hyperaccumulators grown on high-Ni soil. To examine the effects of herbivory on Ni-based defences, we subjected Ni-hyperaccumulating plants to either simulated herbivory or live herbivores. For comparison, we also measured concentrations of organic defences (glucosinolates) within damaged and undamaged hyperaccumulator foliage.

MATERIALS AND METHODS

Study species

The Ni hyperaccumulator, *Streptanthus polygaloides* Gray (Brassicaceae), is an annual herb endemic to serpentine chaparral in the western foothills of California's Sierra Nevada (Reeves *et al.*, 1981; Kruckeberg, 1984). It is of economic interest as one of several plants being evaluated for use in phytoremediation and phytomining (Nicks & Chambers, 1995; McGrath, 1998). Ni measurements in tissues from natural populations have ranged from 1100 to 16400 μg g^{-1} d. wt (Reeves *et al.*, 1981). Plants were raised in a glasshouse in Auburn, Lee County, AL, USA, from seeds collected in the Red Hills Management Area (Favre, 1987), near Chinese Camp, Tuolomne County, CA, USA. Seeds were sown on both high-Ni soil (ProMix (Premiere Horticulture Inc., Red Hill, PA. USA) amended with $NiCl_2$ to a total soil Ni concentration of approx. 1000 μg g^{-1} d. wt) and low-Ni soil (unamended ProMix) and plants were watered as necessary after germination.

For comparison, a congeneric non-hyperaccumulator, *Streptanthus insignus* ssp. *insignus* Jepson, was also grown on high-Ni and low-Ni soils. *S. insignus* is an annual species that is usually limited to serpentine soils of the Southern Coast Ranges in California (Hickman, 1993). Seeds were collected from a population growing on serpentine soil west of Panoche Pass in San Benito County, CA, USA. To avoid phenological effects, only plants which were beginning to bolt from the rosette stage (plant stem height 1.0–2.0 dm) were selected for experimentation.

Effects of nickel on glucosinolate concentrations

Aboveground portions of 10 plants of both species growing on high-Ni and low-Ni glasshouse soil were harvested and immediately frozen in liquid N_2. Tissue was lyophilized and ground in a Retsch grinder (F. Kurt Retsch GmbH & Co.KG, Haan, Germany) to pass through a 0.2 mm sieve. Total glucosinolate content was determined by the Pd-complex method (Møller *et al.*, 1985). Lyophylized tissue (30 mg) was twice-extracted with 1 ml boiling methanol (70% v/v) for 20 min. Eluates were combined and methanol was removed in a vacuum at 40°C. Samples were brought up to 2 ml with distilled H_2O and 1 ml of these crude extracts was placed onto mini-columns of DEAE 25 Sepahadex (Pharmacia Biotech, Bucks, UK; treated with × 10 column volume of 2.0 M acetic acid and rinsed with H_2O

until eluate pH equalled that of the rinsate). Once samples had penetrated the gel, columns were washed with 2.0 ml distilled H_2O and eluates discarded. Intact glucosinolates were eluted from columns with 1.8 ml Na_2CO_3 solution (0.1 M, pH 9.0) and were collected in test tubes, each containing 200 µl 1.0 M HCl. The isolated glucosinolate solution (2.5 ml) was added to 1 ml $PdCl_2$ reagent (88 mg $PdCl_2$ and 420 µl concentrated HCl made up to 250 ml with distilled H_2O) in a test tube. After 30 min, absorbance was measured using a Beckman Du 640 spectrophotometer (Beckman Instruments, Fullerton, CA, USA); $\lambda = 425$ nm.

Nickel concentrations of tissues were determined by ashing 0.1 g of ground, dry sample in a muffle furnace at 450°C for 4.5 h (Hue & Evans, 1986) followed by serial digestions with 1 N HNO_3 and 1 N HCl. Samples were filtered and made up to 10 ml with distilled H_2O in volumetric flasks. Samples were analyzed with an IL 251 atomic absorption spectrometer (Instrumentation Laboratory, Lexington, MA, USA); $\lambda = 720$ nm (Emmel *et al.*, 1977).

Effects of herbivory on glucosinolate and nickel contents

A separate set of *S. polygaloides* plants grown on high-Ni soil was subjected to one of two herbivory treatments (simulated herbivory or lepidopteran herbivory) and compared with undamaged plants. Tissue was removed with scissors for the simulated herbivory treatment and the folivorous larvae of the white cabbage butterfly, *Pieris rapae* L. (Lepidoptera, Pieridae) were used for the lepidopteran herbivory treatment. *P. rapae* was selected because it is oligophagous on members of the plant family Brassicaceae and is a common pest of brassicaceous crops (Ohsaki, 1981). Larvae were obtained from local populations feeding on *Brassica oleracea* L. (Brussels sprout and broccoli) plants in Lee County, AL, USA. Initially, one or two larvae were placed onto each plant and more were added at 2–3 d intervals during the course of the experiment. To simulate natural populations, larvae of different instars were used. Plants with *Pieris* larvae were enclosed in herbivore inclusion cages made of wire covered with organdy fabric. A set of undamaged plants was also enclosed in herbivore cages to test for any cage effects. For simulated herbivory treatments, *c.* 50% of the leaf tissue was removed from distal ends. Based on observations of the feeding behaviour of *Pieris* larvae, the apical meristems were also removed. After 8 d, when *Pieris* larvae had caused an amount of damage similar to that inflicted by simulated herbivory, aboveground portions of plants (5 plants per treatment) were harvested and frozen in liquid N_2. After lyophilization, tissue was ground and analyzed for glucosinolate and Ni concentrations (as previously described). To obtain information on the dietary quality of the leaf, total C and N contents (%) of these tissues were determined using a Fisons NA 1500 NCS Analyzer (Fisons Instruments, Milan Italy) (Torbert *et al.*, 1998).

Statistical analysis

Results were analyzed using StatView 5.0 to conduct a one-way analysis of variance (ANOVA) on each dataset (SAS Institute, 1998). Pairwise comparisons of individual treatment means were made using Fisher's Protected Least Significant Difference (PLSD) test (SAS Institute, 1998). Differences were considered significant at $\alpha \leqslant 0.05$. Many glucosinolate assays use sinigrin (prop-2-enyl glucosinolate) to generate standard curves for estimation of mass of glucosinolates per unit tissue. However, since *S. polygaloides* and *S. insignus* plants produce different types of glucosinolates, none of which is sinigrin (Rodman *et al.*, 1981), raw absorbances indicative of total glucosinolate concentrations were analyzed instead of standard curve values.

RESULTS

Effects of nickel on glucosinolate concentrations

Aboveground portions of *S. polygaloides* plants contained 4000 ± 500 µg Ni g^{-1} dry tissue versus only 100 ± 30 µg Ni g^{-1} dry tissue for plants grown on high-Ni and low-Ni soil, respectively (mean ± SE, $n = 10$ for both treatments). Nickel contents of *S. insignus* plants from both high-Ni and low-Ni soil were below detectable limits (< 10 µg g^{-1} dry tissue). Glucosinolate content was significantly higher in the non-hyperaccumulator (*S. insignus*) plants than in the Ni hyperaccumulator (*S. polygaloides*) plants (Fisher's PLSD, $P = 0.016$; Fig. 1a). Soil Ni concentration did not significantly affect glucosinolate contents of either S. *polygaloides* (Fisher's PLSD, $P = 0.316$) or *S. insignus* (Fisher's PLSD, $P = 0.438$; Fig. 1b,c).

Effects of herbivory on glucosinolate and nickel contents

Concentrations of Ni in aboveground tissues of *S. polygaloides* did not differ among treatments (ANOVA: $F_{3,16} = 0.04$, $P = 0.990$; Fig. 2a). Neither damage nor herbivore-inclusion cages affected plant Ni contents (Fig. 2a).

S. polygaloides plants from both herbivore treatments had higher concentrations of glucosinolates than uncaged control plants (Fig. 2b). Clipped plants and *Pieris*-exposed plants contained 31% and 37% more glucosinolates, respectively, than uncaged control plants (Fisher's PLSD, $P = 0.032$ and 0.069, respectively). Herbivore-inclusion cages did not significantly affect glucosinolate concentrations as

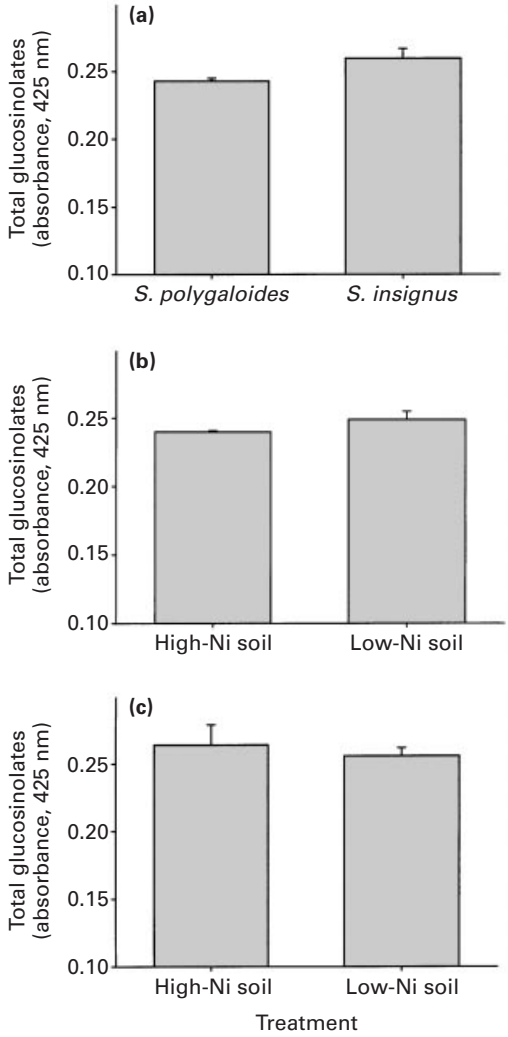

Fig. 1. Total glucosinolate contents of the Ni hyperaccumulator, *Streptanthus polygaloides*, and the non-hyperaccumulator, *S. insignus*, grown on high-Ni and low-Ni soil. (a) *S. polygaloides* and *S. insignus* (high-Ni and low-Ni soil results combined; Fisher's PLSD, $P = 0.016$); (b) comparison of *S. polygaloides* plants grown on high-Ni and low-Ni soil (Fisher's PLSD, $P = 0.316$); (c) comparison of *S. insignus* plants grown on high-Ni and low-Ni soil (Fisher's PLSD, $P = 0.438$). Values are means $\pm$ SE.

caged controls did not differ significantly from uncaged controls (Fisher's PLSD, $P = 0.547$). Interestingly, no differences were detected between the simulated and lepidopteran herbivory treatments (Fisher's PLSD, $P = 0.666$; Fig. 2b).

Effects of herbivory on the dietary quality of the leaf

Total carbon contents (%) of plants from all treatments (Fig. 2c) were similar (ANOVA, $F_{3,16} = 0.75$, $P = 0.541$). Nitrogen concentrations, however, were highest in plants from the two herbivory treatments (Fig. 2d). The N contents of clipped plants were 37% higher than uncaged control plants (Fisher's PLSD, $P = 0.017$) and N concentrations for *Pieris*-exposed plants were 34% higher than the same controls (Fisher's PLSD, $P = 0.025$). Because of these differences in N contents, C:N was lower in both clipped plants and *Pieris*-exposed plants (Fisher's PLSD, $P = 0.015$ and 0.033, respectively) than in uncaged control plants (Fig. 2e). The tissue contents of uncaged and caged controls did not significantly differ in C, N, or C:N (Fisher's PLSD, $P = 0.963$, 0.211 and 0.282, respectively). Likewise, clipped plants and *Pieris*-exposed plants did not differ significantly for these same parameters (Fisher's PLSD, $P = 0.177$, 0.836, and 0.687, respectively; Fig. 2c,d,e).

DISCUSSION

Inducible plant defences require that plant organs be damaged before they are activated or synthesized (Karban & Baldwin, 1997). For *S. polygaloides*, the absence of a response in Ni concentrations to simulated and lepidopteran herbivory suggests that Ni-based defences within this species are not inducible. Before this study, no information has been published specifically addressing the inducibility of Ni-based defences, although damage has been shown to increase Ni concentrations in at least two hyperaccumulator species. In a study examining the feasibility of repeated harvests for phytoremediation, de Varennes *et al.* (1996) showed that aboveground Ni concentrations in *Alyssum pintodasilvae*, a Ni hyperaccumulator, increased in response to clipping. A similar study using the Ni hyperaccumulator, *Berkheya coddii*, showed that new foliage of clipped plants had up to threefold higher Ni contents than the original, excised foliage (Brooks & Robinson, 1998). However, no reference was made to herbivory in either study and the clipping treatments used were modelled after harvesting for phytoextraction purposes and were not designed to mimic herbivore damage.

Experimental evidence suggests that elevated metal contents in plant tissues can defend plants against herbivory (Boyd, 1998). One argument for the selective advantage of metal-based defences is that they are metabolically less expensive than organic defences (Martens & Boyd, 1994; Boyd, 1998). The defensive components (i.e. metals) of metal-based defences are obtained from the soil, therefore the only direct metabolic costs for this type of defence are for the translocation and compartmentalization of metals. Because plants often complex metals with low-molecular-weight organic acids such as citrate (Miyasaka *et al.*, 1991; Sagner *et al.*, 1998), malate (Gabbrielli *et al.*, 1991), histidine (Krämer *et al.*, 1996), oxalate (Mathys, 1977) or phytic acid (Van Steveninck *et al.*, 1990, 1994), the metabolic input required to maintain metal-based defences is considered to be small. It has also been suggested that a 'trade-off' between Ni-based defences and organic defences might exist for

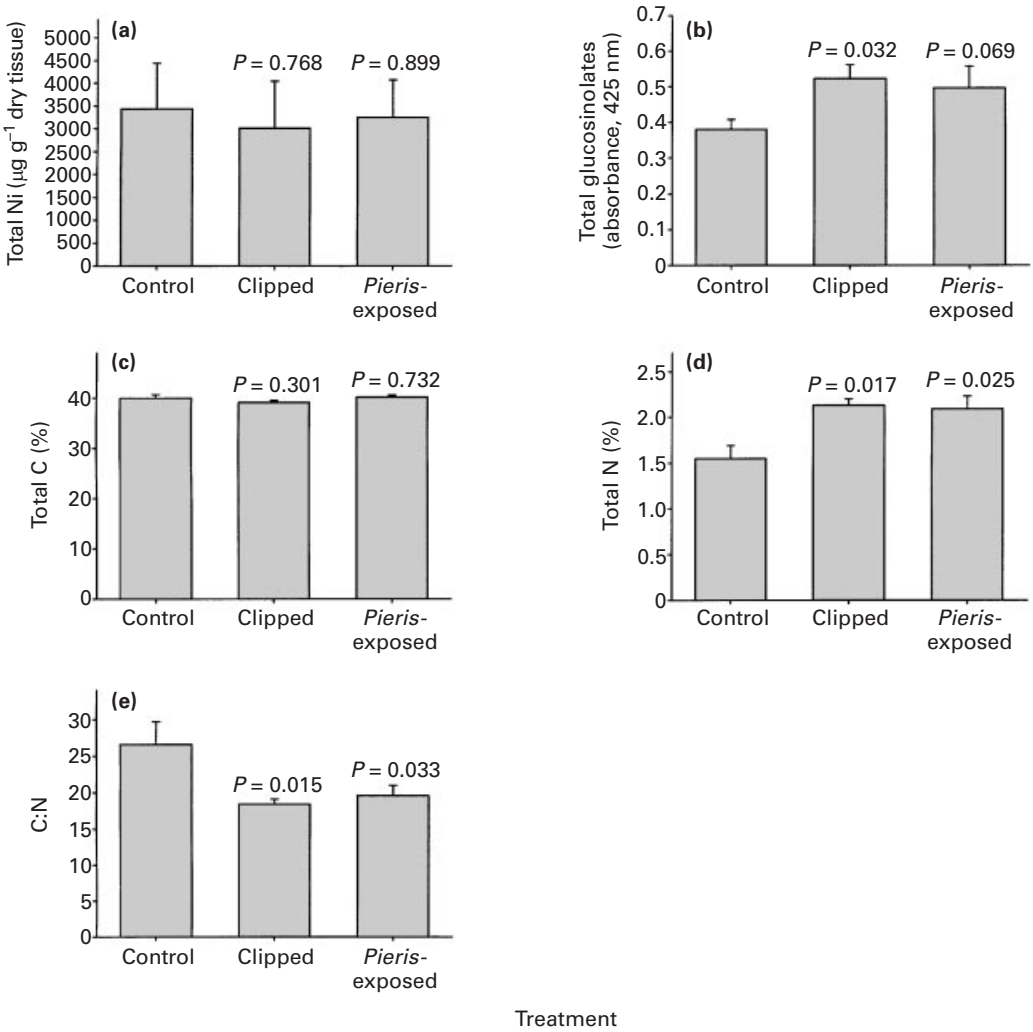

Fig. 2. *Streptanthus polygaloides* herbivory experiment. Bars represent (from left to right): undamaged, uncaged control treatment (Control); simulated herbivory treatment (Clipped); lepidopteran herbivory treatment (*Pieris*-exposed) (see text for details of treatments). (a) Total Ni contents; (b) total glucosinolate contents (absorbance, 425 nm); (c) total C; (d) total N; (e) C : N. *P* values (from Fisher's PLSD tests) indicate a significant difference between herbivory treatment means and uncaged control treatment means; values are means ± SE.

hyperaccumulators (Boyd, 1998). Metal hyperaccumulators might possess lower levels of organic defences than non-hyperaccumulators because their tissues are already well defended by accumulated metal. This hypothesis is supported by our data as the congeneric non-hyperaccumulator (*S. insignus*) had higher glucosinolate concentrations than the Ni hyperaccumulator (*S. polygaloides*).

Levels of many plant defences are not static and are subject to edaphic, climatic, and biotic influences (Coley *et al.*, 1985; Bryant *et al.*, 1987; Ruohomäki *et al.*, 1996; Karban & Baldwin, 1997). Here, the presence of Ni within the hyperaccumulator, *S. polygaloides*, was dependent on Ni availability in the soil, whereas Ni concentrations in *S. insignus* were unaffected by soil Ni concentrations. Contrary to our prediction, glucosinolate contents of both species in this study were unaffected by soil Ni concentrations (Fig. 1a). Because the presence or absence of defensive Ni in *S. polygaloides* did not affect glucosinolate concentrations, any 'trade-off' between organic and Ni-based defences observed between *S. polygaloides* and *S. insignus* is likely to be constitutive and not affected by edaphic conditions. Like *S. polygaloides*, most hyperaccumulators are endemic to serpentine (high metal) soils so it is difficult to demonstrate true evolutionary 'trade-offs' between metal-based and organic defences. Future studies should examine hyperaccumulators that are not serpentine-endemic (e.g. *Thlaspi montanum* var. *montanum*) and that only hyperaccumulate when growing on serpentine soils.

Glucosinolate concentrations often increase in brassicaceous plants that are damaged by herbivory (Bodnaryk, 1992; Bones & Rossiter, 1996; Agrawal, 1998; Hopkins *et al.*, 1998). This was the case in our experiment, as plants from both herbivory treatments contained > 30% higher glucosinolate concentrations than uncaged control plants (Fig. 2b). Some studies have correlated plant sulphur concentrations

with glucosinolate concentrations (e.g. Bones & Rossiter, 1996). Glucosinolates contain N as well as S, and, interestingly, the differences between N concentrations for damaged and undamaged plants from this study (Fig. 2d) mirrored the differences in glucosinolate concentrations (Fig. 2b). We suggest that the increased N in damaged plants might have been owing to the increased production of glucosinolates by those plants.

Many plant defence studies have used simulated herbivory treatments (e.g. scissors, holepunch, needle) to simulate natural herbivory. Although such treatments might adequately model natural herbivory in terms of the percentage of leaf tissue damaged, they might not accurately mimic spatial and temporal patterns of herbivory. In addition, the absence of herbivore saliva and reduction of lepidopteran internal leaf surface area damaged (i.e. multiple chewing sites vs single cut from scissors) might also alter plant responses to simulated herbivory (Alborn *et al.*, 1997; Agrawal, 1998). Thus, some studies have shown that simulated herbivory does not elicit the same response as lepidopteran herbivory (Agrawal, 1998). It is noteworthy that simulated herbivory and lepidopteran herbivory elicited equivalent responses in all parameters measured in this study.

Hyperaccumulation has evolved many times, and in widely spaced geographic locations (Brooks, 1987); hence it is possible that multiple ecological functions exist for hyperaccumulated metals (Boyd & Martens, 1992, 1998). Nickel hyperaccumulators are taxonomically diverse and are present on every vegetated continent (Brooks, 1998). At least 320 species have been reported from 43 plant families (Reeves *et al.*, 1999), with centres of diversity occurring in temperate (Europe), subtropical (Cuba), and tropical regions (New Caledonia). Growth forms of Ni hyperaccumulators vary greatly and range from annuals (e.g. *Streptanthus polygaloides*) to medium-sized (*c.* 15 m) trees (e.g. *Sebertia acuminata*). This diversity makes it difficult to generalize about the selective value of metal hyperaccumulation. Regional taxonomic diversity of Ni hyperaccumulators, however, is sometimes limited. For instance, Ni hyperaccumulators in North America and Europe are mostly in the Brassicaceae (Brooks, 1998), whereas most members of Cuba's Ni-hyperaccumulating flora belong to the Euphorbiaceae and Buxaceae (Reeves *et al.*, 1996, 1999). It is likely, therefore, that accumulated metals within closely related hyperaccumulator species will have a similar function. Thus, the accumulated Ni within *S. polygaloides* and other brassicaeous Ni hyperaccumulators in temperate zones might be defensive, whereas accumulated Ni in the 28 hyperaccumulating species of *Leucocroton* in Cuba (Reeves *et al.*, 1999) might have a different function (e.g. metal tolerance).

Once the ecological functions of metal hyperaccumulation have been determined, the selective pressures that propelled the evolution of this unique ability might be more fully investigated. We suggest that these selective forces (and therefore ecological significance of hyperaccumulation) might differ among geographically and taxonomically disjunct serpentine communities. Further investigations into the ecological function of metals within different hyperaccumulating taxa are needed to clarify the selective value of this ecologically and physiologically unique trait.

ACKNOWLEDGEMENTS

We thank Antonio Motta for assisting with carbon and nitrogen analysis, Dr John Odom for assistance with ICAP and AA analysis, Dr Robert Locy for the use of his laboratory facilities, and Jennifer Borgo for technical assistance. We express gratitude to Dr Roland R. Dute for critically reviewing this manuscript.

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